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Effect of Carbon and Nitrogen Source on the Production of Amylase Enzyme by *Bacillus* spp. under Solid State Fermentation

Keywords: *Amylase, Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus subtilis and Cassava waste.*

Starch-degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food, fermentation, textile to paper industries. The amylases can be derived from several sources such as plants, animals and microbes. The microbial amylases meet industrial demands; a large number of them are available commercially; and, they have almost completely replaced chemical hydrolysis of starch in starch processing industry (Sen et al., 2014). The major advantage of using microorganisms for production of amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics.

Presently the world market for enzymes is about US$ 2.7 Billion and increases with the rate of 4% annually (Abd-Elhalem *et al*.,2015). Now a day's mostly amylase enzymes are produced from *Bacillus* bacterial species. Large production of enzyme can be achieved by media engineering and supplementation of different nutrients which provides good growth for microbes and they produce better enzyme, So, proper optimized medium for the enzyme production is very important for the bacterial culture to produce large amount of enzymes (Abdel-Fattah *et al*., 2013). Different carbon and nitrogen sources like organic and inorganic nitrogen sources are basic need for the enzyme production. With these nutrients, its proper amount is also important for better enzyme production (Deb *et al*., 2013). In this work, medium supplements, Carbon sources and Nitrogen sources (organic nitrogen and inorganic nitrogen) at different concentration was checked and optimized for the large and better amylase enzyme production.

Solid state fermentation (SSF) gained much interest during the past recent years and considered as an economical alternative for enzyme production and application (Govarthanan *et al*., 2015). Industrial development for the utilization of cassava waste has been reviewed bioconversion of cassava waste into protein, biomolecules, organic acids, and food compounds etc. Therefore, the main objectives of the study were to isolate and identify amylolytic bacteria from cassava waste dumpsites, and to perform partial characterization of the enzyme production and its properties with regard to the effect of different carbon and nitrogen sources.

MATERIAL AND METHODS

Substrates used

Cassava waste (Solid waste) was obtained from Sago processing industry, Salem, Tamil Nadu, India.

Isolation of *Bacillus* spp.

Amylolytic bacteria will be isolated from the Cassava solid waste (Cassava baggase). Bacterial isolation will be carried out by serial dilution method on nutrient agar plates. In this case, 0.1 ml of dilution from 10^{-5} of the sample will be inoculated on NA medium for bacteria respectively. The inoculated media will be incubated at 37°C for 24 h. Discrete colonies that developed on the plates will be counted

and recorded as colony forming unit per millilitre (cfu ml^{-1}). The isolates will be further sub-cultured on to slants to obtain the pure culture. Slants will be maintained at 4°C.

Screening and identification of amylase producer

Isolated bacterial colonies, maintained at 4°C, will be further screened for evaluating their amylolytic potential by inoculating them in a starch agar plate. Inoculated plates will be incubated at 37°C for 3 days. After 3 days of incubation amylolytic bacteria will be identified by flooding the plates with Gram's iodine solution (1 g of iodine crystals and 2.0 g of potassium iodide will be dissolved in 100 ml of distilled water, stored at room temperature). (Pokhrel *et al*., 2013).

Characterization of bacterial isolates

Starch reacted with iodine to form a dark blue starch-iodine complex that covered the entire agar. The positive colonies demonstrated a region of clear zone of hydrolysis around the colonies when flooded with grams iodine solution. The negative colonies showed no zone of hydrolysis around them against a blue-black coloration on starch agar. (Benkiar *et al*., 2013 ; Kaur *et al*., 2012; Parmar *et al*., 2012).

Optimization of culture conditions

The effect of culture conditions the present study will be carried out at different carbon sources (Sucrose, Lactose, Maltose, Glucose, Fructose and Mannitol) and different nitrogen sources (Yeast extract, Peptone, Beef extract, Urea, Ammonium chloride and Sodium nitrate) for the aim of producing high quantity of amylase enzyme.

Solid state fermentation

Five gram of substrate (Cassava Solid waste) will be taken into a 250 ml (flask) Erlenmeyer flask and to this a bacterial amylase production containing (g/l) KH_2PO_4 – 1.4; NH_4NO_3 – 10; KCl – 0.5; $MgSO_4.7H_2O$ – 0.1; $FeSO_4.7H_2O$ – 0.01; starch – 20 gm; and distilled water will be added to adjust the require moisture level. The contents of the flasks will be mixed thoroughly and autoclaved at 121°C for 20 min. Solid state fermentation will be carried at 30°C with substrate initial moisture content of 64% for 72 hours using 2 ml of each *Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus subtilis* suspension individually as inoculum. (Castro *et al*., 2010).

Enzyme extraction

22 ml of 0.1 M phosphate buffer (pH-6.5) was added to cultures, the mixture were shacked for 30 min at 19°C and 140 rpm on a rotary shaker. The mixture was filtered through cheese cloth and centrifuged at 8000 rpm at 4°C for 15 min. The supernatant was filtered through Whattman Number-1 filter paper and the filtrate was used as the crude enzyme preparation.

Estimation of amylase activity

a - amylase activity was determined by measuring the rate at which maltose is released from starch which is measured by its ability to reduce 3,5 dinitrosalicylic acid (DNSA). One unit of a - amylase activity was described as the amount of enzyme that will release 1mg of maltose per minute at 25°C. The substrate, 1% cassava baggase was gelatinized in phosphate buffer. The reaction mixture consisted of 1 ml enzyme solution and 1ml of the substrate, incubated for 5 minutes at 25°C and stopped by adding 1ml of DNSA colour reagent. The mixture was heated in a water bath at 100°C for 5 min, cooled and diluted by adding 10ml of distilled water. The reaction mixture was allowed to stand for 15 min at room temperature

and the optical density read at 540nm. A unit of amylase activity was expressed as:

Enzyme activity (Units/ml) =

$$\frac{\text{mg of maltose released}}{\text{Volume of enzyme taken(1 ml)} \times \text{Time of incubation}}$$

Assay of enzyme activity

Decrease in starch-iodine color intensity

Starch forms a deep blue complex with iodine and with progressive hydrolysis of the starch, it changes to red dish brown. Several procedures have been described by various groups for the quantitative determination of amylase based on this property. This method determines the dextrinizing activity of amylase in terms of decrease in the iodine color reaction.

Plate assay

The plate assay was performed using agar plates amended with starch. The agar plates were prepared amended 2% of starch with 1.5% of agar. After agar solidification, around 10 mm diameter of well was cut out aseptically with the help of cork borer. The well was filled with the culture filtrate and incubated at 37 ℃ for overnight. 1% of iodine solution was over layered on the agar and the observation was made to see the hydrolytic zone around the well. The negative control was maintained by adding sterile water in the separate well.

RESULT AND DISCUSSION

Amylase enzyme were produced by *Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus subtilis* using cassava waste from Sago processing industry, Salem. Singh *et al*., 2015 confirmed the production of amylase enzyme from *Bacillus* isolates through the use of iodine on starch agar medium and the developing of clear zone around bacterial growth indicates for amylase activity.

In this present study, the bacterial isolate showing superior amylase production gave the following preliminary characterization: gram-positive spore forming *bacilli*, approximately one micron in length, with central spores usually smaller than the cell. The *Bacillus* spp. *(B.amyloliquefaciens, B.licheniformis, B.subtilis*) was isolated and characterized by Gram staining, motility test, selective medium and biochemical tests.

Effects of different carbon sources (maltose, glucose, sucrose, lactose, mannitol and fructose) and their concentrations on amylase production, growth and sugar utilization by *Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus subtilis* are summarized in Table-1. As shown in Table-1, none of the tested carbon source enhanced the enzyme production by these *Bacillus* spp. except maltose over control medium. Even amylolytic activity was inversely affected by glucose, fructose and sucrose with the amylase production and was recorded lower than control. Suribabu *et al*. (2014) also found maltose superior than other carbon source tested for amylase production with *Brevibacillus borostelensis* R1 under submerged fermentation.

In this present study, the enzyme production was found maximum at carbon source Maltose (861 Uml^{-1}) recorded by *B. licheniformis* and found minimum at carbon source Mannitol (449 Uml^{-1}) recorded by *B. subtilis* (Table 1).

After optimizing carbon sources, various organic and inorganic compounds, Yeast extract, Peptone, Beef extract, Urea, Ammonium chloride and Sodium nitrate were evaluated as nitrogen source for cell growth and amylase production. Qureshi *et al*., (2012) demonstrated that beef extract enhanced the growth of *Bacillus* sp and Amylase yield. Saleem *et al*., (2014) demonstrated that the fungal strain produced maximum amylase titer when grown in mineral medium containing ammonium sulfate as nitrogen source.

Table 1. Effect of carbon source on amylase production

Carbon Source	Enzyme activity (U/ml)		
	Bacillus amyloliquefaciens	*Bacillus licheniformis*	*Bacillus subtilis*
Sucrose	510	554	515
Lactose	523	565	554
Maltose	808	861	843
Glucose	632	577	562
Fructose	644	653	598
Mannitol	449	525	503

Table 2. Effect of Nitrogen Source on amylase production

Nitrogen Source	Enzyme activity (U/ml)		
	Bacillus amyloliquefaciens	*Bacillus licheniformis*	*Bacillus subtilis*
Yeast extract	630	583	544
Peptone	652	599	582
Beef extract	672	680	624
Urea	627	543	496
Ammonium chloride	572	580	524
Sodium nitrate	477	560	440

Effects of different nitrogen sources and their concentrations on amylase production by *Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus subtilis* are summarized in Table-2.

In the present study, the enzyme production was found maximum at Nitrogen source Beef extract (680 Uml^{-1}) recorded by *B. licheniformis* and found minimum at Nitrogen source Sodium nitrate (440 Uml^{-1}) recorded by *B. subtilis* (Table 2).

CONCLUSION

In the present study, natural bacterial flora of the cassava baggase, collected from Salem agro-based industry were identified for amylase production. A total number of 15 bacterial isolates were tested for extracellular amylase production in starch agar medium, and 5 bacterial isolates were found positive based on appearance of zone of hydrolysis in starch agar plates. All the 5 bacterial isolates obtained by initial screening were quantitatively tested for amylase activity and the bacterial species *Bacillus amyloliquefaciens, Bacillus licheniformis* and *Bacillus subtilis* were utilized for amylase production and cassava waste was used as a substrate under solid state fermentation. The selected bacterial isolate that showed considerable amylase activity can be characterized further for various useful industrial purposes.

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